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# Active immunisation of mice with GnRH lipopeptide vaccine candidates: Importance of T helper or multi-dimer GnRH epitope

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ABSTRACT

Active immunisation against gonadotropin releasing hormone (GnRH) is a potential alternative to surgical castration. This study focused on the development of a GnRH subunit lipopeptide vaccine. A library of vaccine candidates that contained one or more (up to eight) copies of monomeric or dimeric GnRH peptide antigen, an adjuvanting lipidic moiety based on lipoamino acids, and an additional T helper epitope, was synthesised by solid phase peptide synthesis. The candidates were evaluated in vivo in order to determine the minimal components of this vaccine necessary to induce a systemic immune response. BALB/c mice were immunised with GnRH lipopeptide conjugates, co-administered with or without Complete Freund's Adjuvant, followed by two additional immunisations. Significant GnRH-specific IgG titres were detected in sera obtained from mice immunised with four of the seven lipopeptides tested, with an increase in titres observed after successive immunisations. This study highlights the importance of for epitope optimisation and delivery system design when producing anti-hapten antibodies in vivo. The results of this study also contribute to the development of future clinical and veterinary immunocontraceptives.



## 1. Introduction

Our understanding and approaches to block or limit the release of reproductive hormones in an attempt to limit their influence on cancers has progressed to the development of immunotherapies. In spite of low immunogenicity, active immunisation against gonadotropin releasing hormone (GnRH) has received considerable attention because of potential applications in immunocontraception. An active area of research is the development of synthetic GnRH-based vaccines against reproductive hormone-dependent male and female cancers.[1] Studies have shown that immunisation against GnRH can reduce prostate levels of testosterone similar to surgical castration in numerous mammalian models, including mice and humans.[2,3] Immunisation of both males and females against GnRH can have a profound effect on fertility through the reduction in sex steroids and cessation of gametogenesis. Hence, there is a potential for GnRH therapeutics to be used either as a semi-permanent contraceptive or to extend the postnatal anovulatory period.[4,5]

In the 1970s a number of groups attempted to produce antibodies against GnRH by co-administration with Complete Freund's Adjuvant (CFA). However, these attempts were unsuccessful in producing high anti-GnRH antibody titers.[6] Many small molecules like peptides (haptens) are successful immunogens only if they are attached to macromolecules (carriers). It is often necessary to modify these haptens for coupling with carriers to make a stable carrier–hapten complex.[7] Selection of a suitable carrier system, hapten density (hapten:carrier molar ratio), and conjugation methods are critical for developing an optimal vaccine against haptens.[8,9] The first alum-adjuvanted vaccine which was successful in producing antibodies against GnRH was conjugated to a tetanus toxoid carrier.[10] A number of other fusion proteins have been prepared for the production of anti-GnRH antibodies that employ conjugation to diphtheria toxoid (e.g. Improvac®), keyhole limpet hemocyanin (e.g. GonaCon™) or ovalbumin, with many of them available commercially for veterinary use.[11–15] Another recombinant fusion protein consisting of GnRH and diphtheria toxoid was successful in phase I/II clinical trials.[16]

Synthetic subunit vaccine systems eliminate the need for large carrier proteins, which are often associated with adverse effects in the host.[17] Since subunit vaccines are composed of different

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individual components (i.e. epitopes, carriers, adjuvants), they are highly customisable. This makes them valuable tools for developing vaccines against weak immunogens, such as GnRH.[18] Lipopeptides can form a synthetic subunit vaccine as one entity that includes all components (adjuvant, carrier and antigen) required by a successful vaccine, eliminating the need for co-administration with an adjuvant.[19,20]

High antibody titers were generated when multiple copies of a peptide were incorporated into a multiple antigenic peptide system with a branched polylysine core.[20] Immunogenicity was further enhanced by the incorporation of lipoamino acid (LAA) residues with this polylysine system, which led to the development of novel candidates for vaccines against a variety of disease states, including cancer. The lipid moieties in the lipopeptide system serve to mimic the action of bacterial adjuvants such as $Pam_3Cys$ and $Pam_2Cys$.[21] Lipopeptide constructs have been found to mediate significant immunogenic effects without additional adjuvants.[22,23]

The development of a GnRH-based subunit lipopeptide vaccine for use as an immunocontraceptive therapy has previously received attention.[24] In this study, we aimed to define the minimal components necessary for the induction of a systemic immune response against GnRH. The immunogenicity of the GnRH decapeptide (EHWSYGLRPG) was enhanced by incorporating it into the lipopeptide systems.

## 2. Results and discussion

### 2.1. Peptide synthesis

Lipopeptides **1**–**5** (Fig. 1) were synthesised by manual and microwave-assisted solid-phase protocols with Boc chemistry.[25] Lipidic moieties were prepared from their 1-bromoalkanes using the method of Gibbons et al. and were used as a mixture of enantiomers in subsequent synthesis.[26] Amino acid couplings involved an activation of residues with DIPEA in DMF, followed by in situ neutralization in the presence of HATU or HBTU. The carbohydrate core for vaccine construct **2** was synthesised from a D-glucose derivative that bore an adipate linker and four *tert*-butoxycarbonyl protected aminopropyl groups as attachment points for GnRH peptide epitopes.[27,28]

The first series of compounds (**1**–**3**, Fig. 1) contained four copies of the GnRH peptide epitope with three copies of 12-carbon LAAs (C12) separated by glycine spacers (**1** and **2**) or 16-carbon LAAs (C16) (**3**) attached to either a poly-lysine (**1** and **3**) or onto a glucose core (**2**). GnRH-based vaccine candidates bearing $Pam_2Cys$ adjuvanting lipid moiety and T helper epitope derived from the L chain of influenza virus hemagglutinin (GALNNRFQIKGVELKS) was effective in a female mouse model.[29] A similar construct (**4**) consisting of two C16 lipid moieties was therefore used in this study. To investigate the immunogenic potential of the GnRH epitope, four copies of a tandem GnRH dimer were synthesised on a poly-lysine lipopeptide to yield 8 copies of the epitope in total (**5**). The presentation of GnRH was enhanced by replacing the Gly residue at position 6 in the parent peptide sequence with a D-Cys (*p*EHWSYcLRPG[30] and conjugated to a linear T helper lipopeptide (**6′**) using Michael addition (Fig. 2).[31] The site-specific conjugation allowed for both terminal regions of GnRH to display the free amino groups important for immune system recognition (**6**). The lipopeptide was designed to have only two 16-carbon LAAs at the N-terminus, with an additional Ser spacer for improved solubility. These modifications led to improved yields (70%) compared to the original design that incorporated a lipidic tail similar to **3** (4%). In compounds **4**–**6**, serine residues were used as spacers between the lipid moiety and epitopes, a modification that was previously reported to enhance the immune response of lipopeptides.[32]

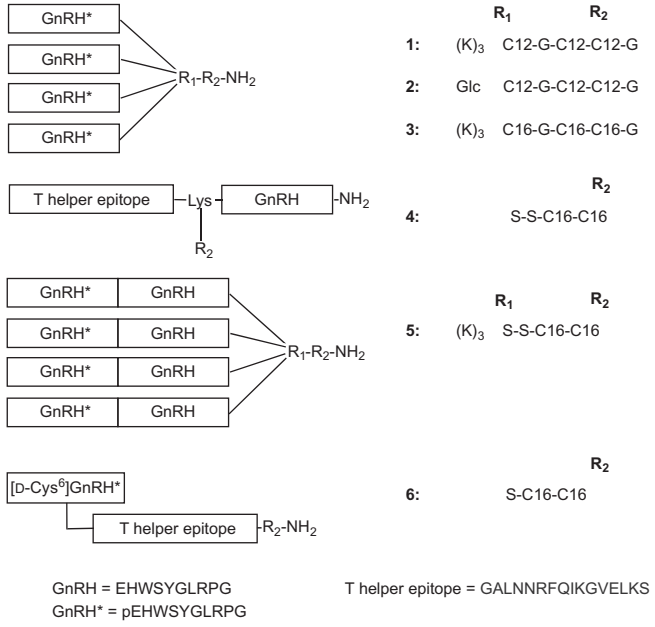


**Figure 1.** Structure of GnRH lipopeptide subunit vaccines (**1**–**6**).

### 2.2. In vitro peripheral blood mononuclear cell (PBMC) toxicity assay

Numerous methods exist to evaluate cellular toxicity; most of them use cell cultures to screen for toxicity by either observing changes in normal cell function or detecting dead cells with stain-based assays. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay has been widely used for determining the cytotoxicity of compounds.[33] We adopted this method with the peripheral blood mononuclear cells (PBMCs) as a model to evaluate the cytotoxicity of the library of lipopeptide vaccine candidates. This model is particularly relevant for testing of drug candidates, and for the assessment of potential toxic risks associated with clinical trials.

In this assay, vaccine candidates **1**–**6** were tested at four rising concentrations (10, 50, 100 and 200 μM). A slight decrease in cell viability was observed by the highest concentration of the vaccine constructs after 48 h; however, it was not statistically significant compared to the negative control (Fig. 3, $p > 0.05$).

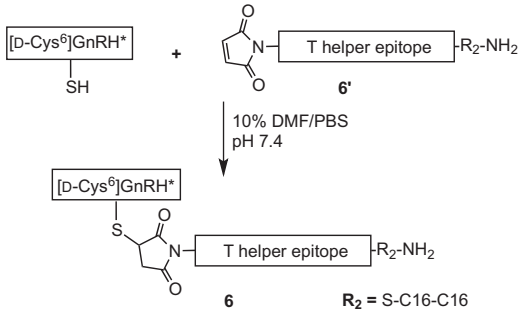


**Figure 2.** Conjugation of [D-Cys$^6$]GnRH* to maleimide conjugated adjuvant (**6′**), to form **6**.

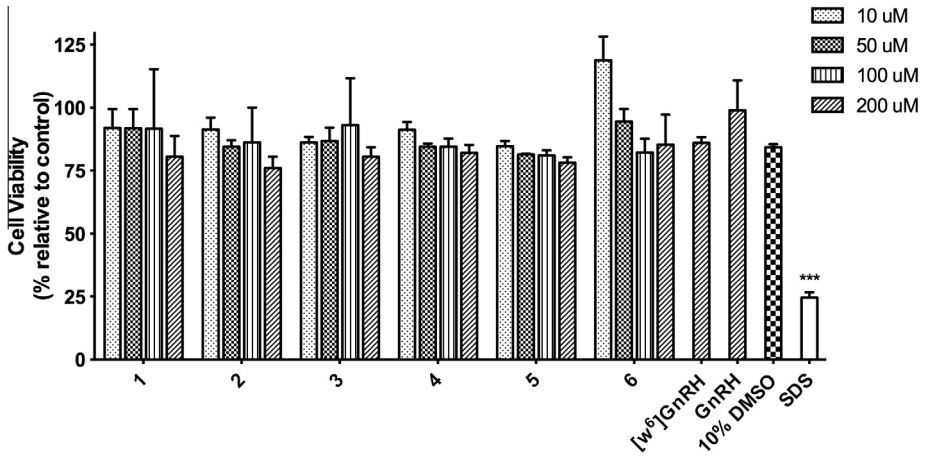


**Figure 3.** Percentage of PBMC viability compared to phosphate-buffered saline (PBS) after 48 h incubation with the lipopeptide vaccine candidates **1**–**6** at four concentrations. Data represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post hoc test and compared to the PBS group.

### 2.3. Murine immunogenicity of synthetic GnRH vaccine candidates

The first study was performed to determine the ability of the GnRH lipopeptides to induce an immune response when four copies of GnRH were incorporated into polylysine or carbohydrate scaffolds (**1**–**3**). Compounds **1**–**3** without T helper epitope were compared to compound **4**, which consisted of one copy of GnRH along with a T helper epitope. All lipopeptide vaccine candidates were dissolved in PBS at a concentration of 1 μg $\mu L^{-1}$ and administered with CFA to adjuvant a significant response to each construct. It was proposed that the immunogenicity of the GnRH hapten would benefit from increased molecular weight, increased copy number (possible B-cell receptor clustering), and the incorporation of a lipid adjuvant. It was anticipated that GnRH linked by the lysine residues of the polylysine core (**1** and **3**) would produce a structure that was large enough to be recognised and processed by the immune system. Since the spatial arrangement may play a part in the immunogenicity of synthetic subunit vaccines, a carbohydrate scaffold was used as a peptide antigen carrier to assemble **2**. However, the first study using compounds **1**–**4** showed that compound **4** administered with CFA was the only compound to produce significant GnRH-specific IgG antibody titers (Fig. 4). This demonstrated that the lipopeptide system alone was insufficient to adjuvant GnRH and required the incorporation of the T helper epitope or another strategy to stimulate a significant immune response in this model. To this end we designed two additional compounds to improve the immunogenicity of the anti-GnRH vaccine construct.

Lipidated GnRH dimers have previously been shown to elicit an antibody response in murine models when administered with CFA.[31] Therefore, in order to increase the possibility of inducing an immune response to GnRH, the peptide was dimerised in tandem and attached to the lipopeptide system in four copies (8 GnRH epitopes in total; **5**). The vaccine **5** co-administered with CFA produced IgG titers comparable to the positive control **4** over the course of the study. Notably, when mice were administered with **5** alone (no CFA), comparable antibody titres ($p$ >0.05) to **5** with CFA were achieved at day 63, (Fig. 5). This demonstrated the adjuvanting activity of the lipopeptide in the construct. It is plausible that dimerization of the GnRH epitope in the construct induced an immune response either due to the formation of small receptor

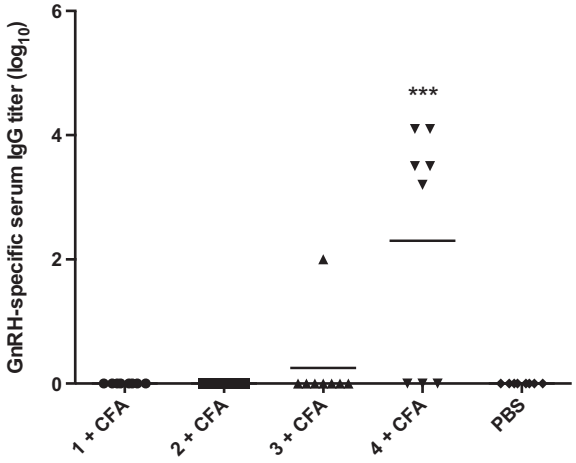


**Figure 4.** GnRH-specific serum IgG titers ($log_{10}$) at day 64 after primary immunisation in response to intramuscular immunisation of BALB/c mice as determined by ELISA of individual sera from the first study (**1**–**4**). Mean IgG titers are represented by a bar. Primary immunisation on day 0 was followed by boosts on days 28 and 56. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test and compared to the PBS group (***$p$ <0.001).

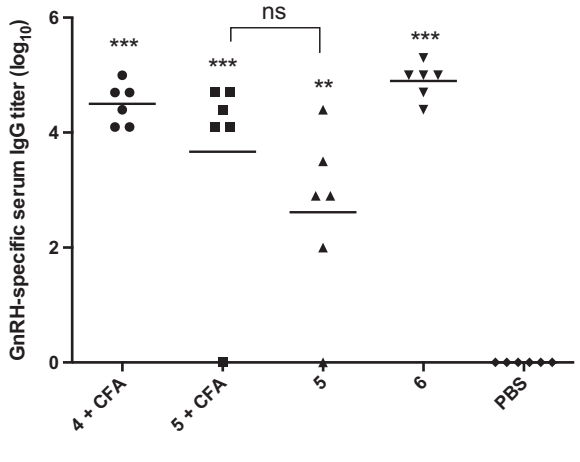


**Figure 5.** GnRH-specific serum IgG titers ($log_{10}$) at day 63 after primary immunisation in response to intramuscular immunisation of BALB/c mice as determined by ELISA for individual sera from the second study (**4**–**6**). Mean IgG titers are presented as a bar. Primary immunisation on day 0 was followed by boosts on days 28 and 56. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test, when each compound was compared to the PBS group (**$p$ <0.01; ***$p$ <0.001) or when **5** with CFA (**5** + CFA) was compared to **5** alone (ns, $p$ >0.05).

clusters co-localised by binding of polyvalent antigens or by the creation of epitopes in the junction of the dimer.[34] In a previous study multiple copies of GnRH was added to a fragment of human IgG and T helper epitope derived from measles virus. It was shown that a significantly greater antibody response was elicited compared to that with same sequence but only a single copy of GnRH epitope.[35]

Interestingly, the vaccine candidate **6** in PBS induced slightly higher GnRH-specific IgG titers in mice than **4** with CFA (Fig. 5). Both constructs consisted of identical principal components (GnRH, T helper epitope and lipid adjuvant), yet arranged in different configurations and conjugated through alternate bonds and positions. Our findings are consistent with a previous study where thioester linkages elicited higher antibody titers when compared to a library of alternative bonds.[36] It was suggested that different spatial arrangements of antigen, carrier, and adjuvant, and the presence of different bonds were responsible for variation in antibody titers.

## 3. Conclusion

This study outlines the synthesis, cytotoxicity and immunogenicity of a library of novel lipopeptide subunit vaccines for GnRH. Self-adjuvanting lipopeptide GnRH vaccine candidates that contained multiple GnRH monomer/dimer epitopes or a thioester-linked GnRH helper epitope system were identified and developed. The investigation of the structure–activity relationship and optimal epitope configuration reinforced that the inclusion of both B and T helper epitopes has a significant effect on systemic anti-GnRH IgG titers. It was also demonstrated that alternative strategies, such as using multiple copies of a peptide dimer, were successful without the need for additional helper epitopes, which can be useful because helper epitope selection can be challenging, particularly when developing a vaccine to be tested on cross-species platforms.[37,38]

This study confirmed the self-adjuvanting activity of the LAA moieties by eliciting comparable anti-GnRH IgG titers to the titers obtained from the constructs co-administered with CFA. Further analysis is necessary to optimise and understand the mechanisms responsible for the immunogenicity of these lipopeptide vaccine candidates and their epitopes. These findings significantly advance out understanding of anti-hapten subunit vaccine design and could contribute to the future development of a commercially viable subunit-based GnRH vaccine.

## 4. Experimental section

### 4.1. Materials and methods

Unless otherwise stated, all chemicals were of analytical grade or equivalent. Acetic anhydride ($Ac_2O$) was supplied by Univar (Ingleburn, NSW, Australia). *N*,*N*-Dimethylformamide (DMF) was obtained from EMD (Darmstadt, Germany). HATU, HBTU, Boc- and Fmoc-L-amino acids, and resins were obtained from Mimotopes (Clayton, VIC, Australia), Novabiochem (Läufelfingen, Switzerland) and Peptides International (Louisville, Kentucky). Di-*tert*-butyldicarbonate was purchased from Auspep (Melbourne, VIC, Australia). Acetic acid (AcOH), *N*,*N*-dichloromethane (DCM), diethyl ether, diisopropylethylamine (DIPEA), HCl, hydrogen bromide in AcOH, ninhydrin, triethylamine (TEA) and trifluoroacetic acid (TFA) were supplied by Merck (Kilsyth, VIC, Australia). HPLC and MS grade acetonitrile (MeCN) and methanol (MeOH) were supplied by Scharlau (Port Adelaide, SA, Australia). Anhydrous hydrofluoric acid (HF) was supplied by BOC gases (Sydney, NSW, Australia). For in vivo experiments, Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and PBS were obtained from GIBCO (Mulgrave, VIC, Australia). Isopropanol was sourced from Lab-Scan Pty Ltd (Dublin, Ireland). All other reagents were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia).

A Kel-F HF apparatus (Peptide Institute, Osaka, Japan) was used for HF cleavage. Thin layer chromatography (TLC) was carried out on Kieselgel 60 F254 silica gel coated aluminum plates (Merck, Darmstadt, Germany). All TLCs were visualised by 20% $H_2SO_4$ or *p*-anisaldehyde followed by heating. Tetrahydrofuran (THF), DCM and $CHCl_3$ were dried by distillation with sodium/benzophenone, calcium hydride and calcium chloride, respectively, then used immediately or stored with 4 Å molecular sieves.

$^1$H NMR spectra were recorded on a BRUKER Avance spectrometer at 300 MHz or 500 MHz in $CDCl_3$. ESI-MS was performed on a triple quadrupole Perkin–Elmer-Sciex API3000 mass spectrometer using a solvent mixture of A [$H_2O$ + 0.1% AcOH] and B [MeCN/$H_2O$ + 0.1% AcOH]. Data were acquired and analysed using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software.

Reverse-phase-high performance liquid chromatography (RP-HPLC) analysis was carried out using a Shimadzu instrument (Kyoto, Japan), with a Grace Vydac (Columbia, MD, USA) Protein/Peptide column. Analysis was achieved using a gradient of solvent A (99.9% $H_2O$/0.1% TFA) and solvent B (90% MeCN/$H_2O$/0.1% TFA). A gradient of 0–100% solvent B (C4), 40–70% solvent B (C4′), or 40–80% solvent B (C4″) over 30 min was employed and absorbance detected at 214 nm. Crude peptides were then purified using a Waters Delta Prep 600 (Milford, MA, USA) or Shimadzu (LC-20AT, SIL-10A, CBM-20A, SPD-20AV, FRC-10A; 20 mL min$^{-1}$) preparative RP-HPLC with a Grace Vydac C4/C18 column. Separation was achieved using a gradient of solvent A and solvent B at a flow rate of 10 mL min$^{-1}$ detected at 230 nm. All peptides were characterised using ESI-qQTOF on an Applied Biosystems QSTAR Pulsar mass spectrometer (Foster City, CA, USA) or ESI-MS on a triple quadrupole API 3000 (Sciex/Applied Biosystems).

### 4.2. Chemistry

#### 4.2.1. 6-Maleimidocaproic acid[39]

Maleic anhydride (2.94 g, 30 mmol) and 6-aminocaproic acid (3.94 g, 30 mmol) were refluxed in AcOH (70 mL) overnight. $Ac_2O$ (2.83 mL, 30 mmol) was added dropwise and reflux continued for a further 1 h. The acetic acid was co-evaporated with toluene in vacuo to yield yellow syrup. The material was purified over $SiO_2$ (DCM:MeOH, 10:0.7) affording a cream crystalline solid in 57% yield (3.58 g): $R_f$ (DCM/MeOH, 10:0.7) = 0.50; ESI-MS ($C_{10}H_{13}NO_4$, 211.1): $m/z$ = 212.0 [M+H$^+$] (calcd 212.1), 423.1 [M+2H$^+$] (calcd 423.2); $^1$H NMR: $\delta$ = 6.95 (2H, s, CH=CH), 3.35 (2H, t, *J* 7.4 Hz, $NCH_2$), 2.15 (2H, t, *J* 7.2 Hz, $CH_2COO^-$), 1.45 (4H, m, $2CH_2$), 1.18 (2H, m, $CH_2$).

#### 4.2.2. 2-*tert*-Butoxycarbonylaminododecanoic acid lipoamino acid (Boc-C12-OH)[40]

2-Aminododecanoic acid was synthesised as described in the literature with 1-bromodecane and diethyl acetoamidomalonate.[26] The free amine of 2-aminohexadecanoic acid was then Boc-protected by reaction with di-*tert*-butyl dicarbonate in a basic environment as previously reported.[26,41] C12 was prepared to afford the pure product in 54.0% yield (1.31 g): $R_f$ = 0.65 (DCM/MeCN/AcOH); ESI-MS ($C_{17}H_{33}NO_4$, 315.2): $m/z$ = 316.4 [M+H]$^+$ (calcd 316.2), 338.2 [M+Na]$^+$ (calcd 338.2); $^1$H NMR: $\delta$ = 4.94–4.92 (1H, d, *J* 7.1 Hz, OCONH), 4.26 (1H, m, α-CH), 1.82–1.67 (2H, m, β-CH), 1.42 (9H, s, $C(CH_3)_3$), 1.23 (16H, br s, $8CH_2$), 0.86 (3H, t, *J* 7.0 Hz, $CH_3$).

#### 4.2.3. 2-*tert*-Butoxycarbonylaminohexadecanoic acid lipoamino acid (Boc-C16-OH)[42]

2-Aminohexadecanoic acid was synthesised as described in the literature with 1-bromotetradecane and diethyl acetoamidomalonate. The free amine of 2-aminohexadecanoic acid was then Boc-protected by reaction with di-*tert*-butyl dicarbonate in a basic environment as previously reported.[26] The C16 product was prepared to yield (78%) the pure product (1.89 g): $R_f$ = 0.68 (DCM/MeCN/AcOH); ESI-MS ($C_{21}H_{41}NO_4$, 371.6): $m/z$ = 394.3 $[M+Na]^+$ (calcd 374.6); NMR: $\delta$ = 4.92 (1H, m, OCONH), 4.26 (1H, m, α-CH), 1.93–1.58 (2H, m, *J* 7.5 Hz, β-CH), 1.45 (9H, s, $C(CH_3)_3$), 1.25 (24H, br s, $12CH_2$), 0.86 (3H, t, *J* 6.9 Hz, $CH_3$).

#### 4.2.4. 2-((1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl)amino)hexadecanoic acid (Dde-C16-OH)[43]

5,5-Dimethyl-1,3-cyclohexanedione (2.5 g, 17.8 mmol) was dissolved in DCM (15 mL). 4-Dimethylaminopyridine (435 mg) and TEA (5 mL) were then added and the mixture was stirred for 10 min. $Ac_2O$ (2.2 mL) was added and the mixture was stirred under Ar for 2 days. The solvent was removed in vacuo by co-evaporation of the crude product in ethyl acetate (EtOAc) (50 mL) with toluene and then washed with 5% HCl (3 × 50 mL) and dried with $MgSO_4$. The solvent was evaporated to produce an oil which was filtered through a column of silica (Hex/EtOAc, 3:2). The EtOAc from the mixture obtained was evaporated in vacuo to afford an orange oil, which was then cooled to yield pale yellow crystals (2-acetyldimedone, Dde-OH) in 72% yield (2.35 g): $R_f$ (Hex:EtOAc, 3:2) = 0.76; ESI-MS ($C_{10}H_{15}NO_2$, 181.1): $m/z$ = 182.2 $[M+H]^+$ (calcd 182.1); $^1$H NMR: $\delta$ = 2.59 (s, 3H, C=C$(CH_3)_2$) ; 2.52, 2.35 (2s, 4H, $2CH_2$) ; 1.06 (s, 6H, $2CH_3$).

2-Amino-D,L-hexadecanoic acid hydrochloride (2 g, 7.48 mmol) and Dde-OH (1.5 g, 8.23 mmol) were suspended in ethanol (30 mL). TEA (2.6 mL) was added and the mixture refluxed under an inert atmosphere for 2 days. The solvent was evaporated and the crude product taken up in EtOAc (50 mL) and washed with 5% HCl (3 × 30 mL) then dried over $MgSO_4$. The solvent was evaporated to afford a solid which was triturated with diethyl ether to afford the pure product as a white solid in 55% yield (1.78 g): $R_f$ ($CHCl_3$/MeOH, 10:0.7) = 0.58; ESI-MS ($C_{26}H_{45}NO_4$, 435.3): $m/z$ = 436.4 $[M+H]^+$ (calcd 436.3); $^1$H NMR: $\delta$ = 4.38 (1H, dd, *J* 6.3, 6.6 Hz, α-CH), 2.52 (3H, s, C(NH)$CH_3$), 2.40 (4H, s, $2CH_2CO$), 2.04–1.90 (2H, m, β-$CH_2$), 1.46–1.23 (24H, m, $12CH_2$), 1.02 (6H, s, $C(CH_3)_2$), 0.86 (3H, t, *J* 6.9 Hz, $CH_3$).

#### 4.2.5. Synthesis of lipopeptides

All peptides were synthesised using standard manual solid phase peptide synthetic protocols and then purified by RP-HPLC. Lipopeptides **1**-**5** were synthesised using *p*-4-methyl benzhydryl amine (*p*-MBHA; substitution 0.45 mmol $g^{-1}$) resin (Peptides International, USA), which was swollen in DMF/DIPEA for approximately 1 h. Synthesis was carried out using 4 equiv Boc-L-amino acids. Lipopeptides were synthesised using 8 equiv of activated amino acids after the addition of the second Lys residue. The peptides were then coupled using amino acids preactivated with an equimolar amount of 0.5 M HBTU or HATU in DMF and 6 equiv DIPEA, then mixed with the resin for 30–45 min at rt or for 5–10 min at 70 °C, 20 W. For Boc chemistry synthesis, Boc protecting groups were used for the α-amino-termini; Tos for Arg; DNP and Bom for His; Bzl for Ser; For for Trp; 2-Br-Z for Tyr; 2Cl-Z for Lys; Xan for Asn and Gln; and OcHx for Glu. Boc-C12-OH and Boc-C16-OH were used to assemble the N-terminal lipid adjuvant. Boc-Lys(Boc)-OH·DCHA salt was neutralised using 0.5 M $NaHSO_4$/EtOAc, dried in vacuo and utilised for the synthesis of the polylysine carrier. The Boc protecting groups were removed from the amino acids using neat TFA. Prior to final Boc deprotection, the formyl/Fmoc and DNP protecting groups were removed with 20% piperidine in DMF and 20% 2-mercaptoethanol/10% DIPEA in DMF, respectively. The resin was washed with DMF after each manipulation. After the last coupling, all terminal Boc and Fmoc groups were removed. The resin was then washed consecutively with DMF, DCM and MeOH and left to dry in vacuo overnight. The peptides were cleaved from the resin using the high HF method at a concentration of 10 mL $g^{-1}$ of resin and 5% *p*-cresol at −5 to 0 °C for 1–2 h. Following this, the peptide was precipitated in diethyl ether, washed through a polyethylene frit, then dissolved in 1:1 MeCN/$H_2O$ and lyophilised.

Synthetic adjuvants for conjugation to [D-Cys$^6$]GnRH were assembled using microwave-assisted Fmoc solid phase peptide synthetic protocols. The peptides were synthesised on Rink Amide MBHA LL (100–200 mesh, 0.34 mmol $g^{-1}$) resin (Peptides International, USA), which was swelled in DMF/DIPEA for approximately 15 min. Peptides were then coupled using amino acids that had been activated for 1 min with an equimolar amount of 0.5 M HATU in DMF and 5 equiv DIPEA, then mixed with the resin for 10 min at 70 °C, 20 W. The coupling efficiencies were monitored using the ninhydrin test calculated by reading the absorbance at 570 nm. If the coupling efficiency was below 99.6%, the coupling was repeated until the required efficiency was achieved. For Fmoc chemistry synthesis, Fmoc protecting groups were used for the α-amino-termini; Pbf for Arg; Trt for Asn, Cys, His, and Gln; *t*Bu for Ser; Boc for Trp and Lys; and O*t*Bu for Glu. Dde-C16-OH was used to assemble the N-terminal lipid adjuvant. The Fmoc protecting groups were removed from the amino acids using 20% piperidine/DMF. Dde groups were removed using 2% hydrazine hydrate in DMF. After each manipulation the resin was washed with DMF. After the last coupling, the resin was washed consecutively with DMF, DCM and MeOH then left to dry in vacuo overnight. The peptides were cleaved from the resin using 95% TFA/2.5% $H_2O$/2.5% TIPS (10–25 mL $g^{-1}$) for 2 h at rt. Following this, the slurry was dried, precipitated in diethyl ether, then dissolved in 1:1 MeCN/$H_2O$ and lyophilised.

Compound **1**; purified yield: 5.0 mg, 23.3%; HPLC: $t_R$ (C4) = 20.60 min; ESI-MS ($C_{278}H_{402}N_{76}O_{60}$, 5768.6): $m/z$ = 825.4 $[M+7H]^+$ (calcd 825.1), 962.7 $[M+6H]^+$ (calcd 962.4), 1154.8 $[M+5H]^+$ (calcd 1154.7), 1444.0 $[M+4H]^+$ (calcd 1443.2), 1923.8 $[M+3H]^+$ (calcd 1923.9).

Compound **2**; purified yield: 5.8 mg, 26%; HPLC: $t_R$ (C4) = 20.63 and 21.47 min; ESI-MS ($C_{284}H_{413}N_{75}O_{64}$, 5901.8): $m/z$ = 846.4 $[M+7H]^{7+}$ (calcd 844.1), 984.5 $[M+6H]^{6+}$ (calcd 984.5), 1181.4 $[M+5H]^{5+}$ (calcd 1181.2).

Compound **3**; purified yield: 5.0 mg, 25%; HPLC: $t_R$ (C4) = 21.27 min; $t_R$ (C4′) = 20.10 min; ESI-MS ($C_{290}H_{426}N_{76}O_{60}$, 5936.3): $m/z$ = 849.4 $[M+7H]^{7+}$ (calcd 849.0), 990.5 $[M+6H]^{6+}$ (calcd 990.5), 1188.9 $[M+5H]^{5+}$ (calcd 1188.3), 1485.1 $[M+4H]^{4+}$ (calcd 1485.1).

Compound **4**; purified yield: 2.0 mg, 56%; HPLC: $t_R$ (C4) = 32.34 min; ESI-MS ($C_{179}H_{291}N_{47}O_{44}$, 3805.51): $m/z$ = 636.4 $[M+6H]^{6+}$ (calcd 635.2), 763.1 $[M+5H]^{5+}$ (calcd 762.1), 953.2 $[M+4H]^{4+}$ (calcd 952.3), 1270.9 $[M+3H]^{3+}$(calcd 1269.5), 1905.8 $[M+2H]^{2+}$ (calcd 1903.7).

Compound **5**; purified yield: 8.0 mg, 8%; HPLC: $t_R$ (C4) = 22.00, 22.37 min; $t_R$ (C4′) = 20.90, 22.40 min; ESI-MS ($C_{496}H_{695}N_{139}O_{117}$, 10475.3): $m/z$ = 953.7 $[M+11H]^{11+}$ (calcd 953.3), 1049.1 $[M+10H]^{10+}$ (calcd 1048.5), 1165.2 $[M+9H]^{9+}$ (calcd 1164.9), 1311.1 $[M+8H]^{8+}$ (calcd 1310.4), 1498.2 $[M+7H]^{7+}$ (calcd 1497.5), 1747.7 $[M+6H]^{6+}$ (calcd 1746.9).

#### 4.2.6. Thioester conjugation of [D-Cys$^6$]GnRH to maleimide adjuvant (6)

The maleimide-T helper peptide adjuvant system, **6**′ (2.0 mg), was dissolved in DMF (2 mL). Reduced (by 10 equiv tris(2-carboxyethyl)phosphine, then purified) [D-Cys$^6$]GnRH (2.0 mg) in PBS (18 mL) was then added, the reaction degassed, and left to stir at

rt under $N_2$ for 5 h. The product (**6**) was then purified using RP-HPLC and characterised with ESI-MS.

Compound **6′**; purified yield: 88 mg, 95%; HPLC: $t_R$ (C4) = 24.20 min; ESI-MS ($C_{123}H_{211}N_{29}O_{29}$, 2559.6): $m/z$ = 1281.0 $[M+2H]^{2+}$ (calcd 1281.3), 854.3 $[M+3H]^{3+}$ (calcd 854.9).

Compound **6**; purified yield = 2.1 mg, 70%; HPLC: $t_R$ (C4) = 22.32 min; $t_R$ (C4′) = 15.60, 15.93 min; ESI-MS ($C_{179}H_{288}N_{46}O_{42}S$, 3788.2): $m/z$ = 758.6 $[M+5H]^{5+}$ (calcd 758.6), 947.9 $[M+4H]^{4+}$ (calcd 948.1), 1264.0 $[M+3H]^{3+}$ (calcd 1263.7), 1895.4 $[M+2H]^{2+}$ (calcd 1895.1).

### 4.3. PBMC isolation and in vitro cell toxicity assay

Assay was undertaken with the approval from the University of Queensland Ethics Committee (Ethics Approval Number: 2009000661). Blood samples (4 mL) were taken from a healthy adult consented volunteer and PBMCs were isolated by Ficoll gradient after centrifugation at 400*g* for 30 min. The buffy coat layer including mononuclear cells was removed and washed three times with RPMI 1640 (4 mL). After the last washing step, cells were resuspended in 10% FBS:RPMI and seeded at $1 \times 10^6$ cells/mL in a 96-well flat bottom plates (TPP), activated by adding 10 μg $mL^{-1}$ of phytohemagglutinin and incubated at 37 °C in a 5% $CO_2$ atmosphere. After 1 h incubation compounds were added at 10 μL/well in at 10, 50, 100 and 200 μM. MTT (10 μL, 10 mg/mL) (Sigma–Aldrich) was added to each well and incubated for 4 h. 100 μL of acidified isopropanol (0.1 N HCl) was added to dissolve the formazan crystals and left to incubate at 37 °C for 30 min. A Molecular Devices SpectraMax 250 microplate reader (Sunnyvale, CA, USA) was used to measure the absorbance of each well at 570 nm, with background subtracted at 690 nm. The percentage of viable cells for each compound was calculated by comparing absorbance with PBS positive control. SDS was used as a negative control.

### 4.4. Immunisation protocol

All protocols were approved by The University of Queensland Animal Ethics Committee (AEC#SCMB/005/11/ARC) and carried out according to their guidelines. Female BALB/c mice were used for immunisation. Mice ($n$ = 6–8/group) received a primary intramuscular (im) dose of 50 μg of immunogen in a total volume of 50 μL of PBS. Two boosts (same as the primary dose) were administered to mice at days 28 and 56. The negative control group was administered 50 μL of PBS and the positive control group received one dose of 50 μg of immunogen in a total volume of 50 μL 1:1 CFA:PBS, followed by boosts in 50 μL PBS alone. Blood (10 μL ea.) was collected from tail snips of each mouse. The blood was left in 90 μL PBS at rt for ~30 min and centrifuged at 3000 rpm for 10 min to pellet red blood cells. Serum was stored at −20 °C.

#### 4.4.1. Detection of systemic IgG by ELISA

Flat-bottom microtiter plates (Immulon™ 2HB, Thermo Scientific, USA) were coated with 100 μL/well of a solution of streptavidin (Sigma–Aldrich) (5 μg $ml^{-1}$) in $dH_2O$ at 37 °C on a shaker/incubator overnight to dryness. A 4% BSA solution (150 μL) in 0.05% Tween-20/PBS was added and incubated for 1 h at 37 °C. Plates were washed with PBS/Tween-20 (0.05%) and 100 μL of peptide (5 μg $ml^{-1}$ [Biotin-Lys$^{11}$]GnRH) in 0.05% Tween-20/0.1% $NaN_3$/PBS was added for 1 h at 37 °C. Plates were washed and murine sera at a 1/100 dilution (continued with a 1:2 dilution down the plate) in 1% BSA/PBS (100 μL/well) was added and incubated for 1.5 h at 37 °C in a humidified atmosphere. The sera were removed, the plates washed and 100 μL of a 1/3000 dilution of HRP-conjugated goat anti-mouse IgG (Jackson Laboratories/BioRad, USA) in 1% BSA/PBS was added. After 1 h at 37 °C, the plates were washed and 100 μL of *o*-phenylenediamine dihydrochloride substrate (SigmaFast, Sigma–Aldrich) was added. Plates were incubated in the dark for 30 min and the absorbance of the solutions was then determined at a wavelength of 450 nm using a Molecular Devices SpectraMax 250 microplate reader. Cut-off was determined as the mean of PBS antisera plus 3 standard deviations.

#### 4.4.2. Statistics

Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test. GraphPad Prism 5 software was used for statistical analysis, with $p$ <0.05 taken as statistically significant.

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## References and notes

1. Talwar, G. P. *Immunol. Rev.* **1999**, *171*, 173.
2. Raina, K.; Panda, A. K.; Ali, M. M.; Talwar, G. P. *Protein Expr. Purif.* **2004**, *37*, 8.
3. Junco, J. A.; Peschke, P.; Zuna, I.; Ehernann, V.; Fuentes, F.; Bover, E.; Pimentel, E.; Basulto, R.; Reyes, O.; Calzada, L.; Castro, M. D.; Arteaga, N.; Lopez, Y.; Garay, H.; Hernandez, H.; Bringas, R.; Guillen, G. E. *Vaccine* **2007**, *25*, 8460.
4. Ferro, V. A.; Khan, M. A. H.; Earl, E. R.; Harvey, M. J. A.; Colston, A.; Stimson, W. H. *Am. J. Reprod. Immunol.* **2002**, *48*, 361.
5. Fraser, H. M.; Dewart, P. J.; Smith, S. K.; Cowen, G. M.; Sandow, J.; McNeilly, A. S. *J. Clin. Endocrinol. Metabol.* **1989**, *69*, 996.
6. Talwar, G. P. *J. Steroid Biochem. Mol. Biol.* **1985**, *23*, 795.
7. Rajesh, K.; Rana, K. V.; Suri, C. R. *BioNanoScience* **2013**, *3*, 137.
8. Pedersen, M. K.; Sorensen, N. S.; Heegaard, P. M. H.; Beyer, N. H.; Bruun, L. *J. Immunol. Methods* **2006**, *311*, 198.
9. Bachmann, M. F.; Rohrer, U. H.; Kundig, T. M.; Burki, K.; Hengartner, H.; Zinkernagel, R. M. *Science* **1993**, *262*, 1448.
10. Shastri, N.; Manhar, S. K.; Talwar, G. P. *Am. J. Reprod. Immunol.* **1981**, *1*, 262.
11. Jayashankar, R.; Chaudhuri, M. K.; Singh, O.; Alam, A.; Talwar, G. P. *Prostate* **1989**, *14*, 3.
12. Giri, D. K.; Jayaraman, S.; Neelaram, G. S.; Jayashankar, R.; Talwar, G. P. *Exp. Mol. Pathol.* **1991**, *54*, 255.
13. Miller, L. A.; Gionfriddo, J. P.; Fagerstone, K. A.; Rhyan, J. C.; Killian, G. J. *Am. J. Reprod. Immunol.* **2008**, *60*, 214.
14. Dunshea, F. R.; Colantoni, C.; Howard, K.; McCauley, I.; Jackson, P.; Long, K. A.; Lopaticki, S.; Nugent, E. A.; Simons, J. A.; Walker, J.; Hennessy, D. P. *J. Anim. Sci.* **2001**, *79*, 2524.
15. Ulker, H.; Kucuk, M.; Yilmaz, A.; Yoruk, M.; Arslan, L.; DeAvila, D. M.; Reeves, J. J. *Reprod. Domest. Anim.* **2009**, *44*, 37.
16. Parkinson, R. J.; Simms, M. S.; Broome, P.; Humphreys, J. E.; Bishop, M. C. *Eur. Urol.* **2004**, *45*, 171.
17. Babiuk, L. A. *Vaccine* **1999**, *17*, 1587.
18. Moyle, P. M.; Toth, I. *ChemMedChem* **2013**, *8*, 360.
19. Moyle, P. M.; McGeary, R. P.; Blanchfield, J. T.; Toth, I. *Curr. Drug Delivery* **2004**, *1*, 385.
20. Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409.
21. Moyle, P. M.; Toth, I. *Curr. Med. Chem.* **2008**, *15*, 506.
22. Olive, C.; Batzloff, M. R.; Toth, I. *Exp. Rev. Vaccines* **2004**, *3*, 43.
23. Olive, C.; Ho, M. F.; Dyer, J.; Lincoln, D.; Barozzi, N.; Toth, I.; Good, M. F. *J. Infect. Dis.* **2006**, *193*, 1666.
24. Goodwin, D.; Simerska, P.; Toth, I. *Curr. Med. Chem.* **2012**, *19*, 4451.
25. Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Res. Ther.* **2007**, *13*, 31.
26. Gibbons, W. A.; Hughes, R. A.; Charalambous, M.; Christodoulou, M.; Szeto, A.; Aulabaugh, A. E.; Mascagni, P.; Toth, I. *Liebigs Ann. Chem.* **1990**, 1175.
27. Simerska, P.; Abdel-Aal, A. B. M.; Fujita, Y.; Moyle, P. M.; McGeary, R. P.; Batzloff, M. R.; Olive, C.; Good, M. F.; Toth, I. *J. Med. Chem.* **2008**, *51*, 1447.
28. Simerska, P.; Lu, H.; Toth, I. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 821.
29. Zeng, W. G.; Ghosh, S.; Lau, Y. F.; Brown, L. E.; Jackson, D. C. *J. Immunol.* **2002**, *169*, 4905.
30. Rafiee, A.; Mansfeld, F. M.; Moyle, P. M.; Toth, I. *Int. J. Org. Chem.* **2013**, *03*, 51.
31. Beekman, N. J. C. M.; Schaaper, W. M. M.; Tesser, G. I.; Dalsgaard, K.; Kamstrup, S.; Langeveld, J. P. M.; Boshuizen, R. S.; Meloen, R. H. *J. Pept. Res.* **1997**, *50*, 357.
32. Chua, B. Y.; Zeng, W.; Lau, Y. F.; Jackson, D. C. *Vaccine* **2007**, *25*, 92.

33. Nikkhah, G.; Darling, J. L.; Thomas, D. G. T. *Anticancer Res.* **1987**, *7*, 902.
34. Hat, B.; Kazmierczak, B.; Lipniacki, T. *PLoS Comput. Biol.* **2011**, *7*, e1002197.
35. Jinshu, X.; Jingjing, L.; Duan, P.; Zheng, Z.; Ding, M.; Jie, W.; Rongyue, C.; Zhuoyi, H. *J. Immunol. Methods* **2004**, *289*, 111.
36. Beekman, N. J. C. M.; Schaaper, W. M. M.; Langeveld, J. P. M.; Boshuizen, R. S.; Meloen, R. H. *J. Pept. Res.* **2001**, *58*, 237.
37. Testa, J. S.; Philip, R. *Future Virol.* **2012**, *7*, 1077.
38. Blanco, E.; Garcia-Briones, M.; Sanz-Parra, A.; Gomes, P.; De Oliveira, E.; Valero, M. L.; Andreu, D.; Ley, V.; Sobrino, F. *J. Virol.* **2001**, *75*, 3164.
39. Lee, N. J.; Kang, N. I.; Cho, W. J.; Kim, S. H.; Kang, K. T.; Theodorakis, E. A. *Polym. Int.* **2001**, *50*, 1010.
40. Blanchfield, J.; Dutton, J.; Hogg, R.; Craik, D.; Adams, D.; Lewis, R.; Alewood, P.; Toth, I. *Lett. Pept. Sci.* **2001**, *8*, 235.
41. Toth, I.; Flinn, N.; Hillery, A.; Gibbons, W. A.; Artursson, P. *Int. J. Pharm.* **1994**, *105*, 241.
42. Skwarczynski, M.; Toth, I. *Methods Mol. Biol.* **2011**, *751*, 297.
43. Ross, B. P.; Falconer, R. A.; Toth, I. *Molbank* **2008**, *2008*.